

GABA ρ 1/GABA α 1 receptor chimeras to study receptor desensitization

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γ -Aminobutyrate type C (GABA $_C$) receptors are ligand-gated ion channels that are expressed preponderantly in the vertebrate retina and are characterized, among other things, by a very low rate of desensitization and resistance to the specific GABA $_A$ antagonist bicuculline. To examine which structural elements determine the nondesensitizing character of the human homomeric ρ 1 receptor, we used a combination of gene chimeras and electrophysiology of receptors expressed in *Xenopus* oocytes. Two chimeric genes were constructed, made up of portions of the ρ 1-subunit and of the α 1-subunit of the GABA $_A$ receptor. When expressed in *Xenopus* oocytes, one chimeric gene (ρ 1/ α 1) formed functional homooligomeric receptors that were fully resistant to bicuculline and were blocked by the specific GABA $_C$ antagonist (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid and by zinc. Moreover, these chimeric receptors had a fast-desensitizing component, even faster than that of heterooligomeric GABA $_A$ receptors, in striking contrast to the almost nil desensitization of wild-type ρ 1 (wt ρ 1) receptors. To see whether the fast-desensitizing characteristic of the chimera was determined by the amino acids forming the ion channels, we replaced the second transmembrane segment (TM2) of ρ 1 by that of the α 1-subunit of GABA $_A$. Although the α 1-subunit forms fast-desensitizing receptors when coexpressed with other GABA $_A$ subunits, the sole transfer of the α 1TM2 segment to ρ 1 was not sufficient to form desensitizing receptors. All this suggests that the slow-desensitizing trait of ρ 1 receptors is determined by a combination of several interacting domains along the molecule.

Xenopus oocytes | γ -aminobutyrate type C receptor | (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid

Several years ago a new type of γ -aminobutyrate (GABA) receptor, now called GABA $_C$, was identified clearly by expressing retina mRNA in *Xenopus* oocytes (1). In sharp contrast to the previously known GABA $_A$ receptors, the GABA $_C$ receptors show very little desensitization, are not blocked by the specific GABA $_A$ receptor antagonist bicuculline, and are not modulated by pentobarbital or steroids (1–3). GABA $_C$ is now recognized as a new family of receptors of which, so far, three members have been cloned (ρ 1– ρ 3). Moreover, there is a larger diversity of GABA $_C$ receptors because of alternative splicing (4). In contrast to the heteromeric nature of GABA $_A$ receptors, which are formed by a combination of several types of subunits, the three GABA $_C$ genes form functional homomeric receptors when expressed in *Xenopus* oocytes (5–9).

The electrical responses to GABA, which have been recorded from various types of retinal cells, are due to activation of both GABA $_A$ and GABA $_C$ receptors (10–14), and, except for a bicuculline-resistant GABA current seen in the golden perch retina (15), all other GABA $_C$ responses desensitize very slowly. For example, the GABA currents elicited in oocytes expressing GABA ρ 1 or its alternative spliced form (GABA ρ 1 Δ 51) decay only about 8% or less even after 10 min of exposure to GABA (4). Although the functional role of GABA $_C$ receptors in the retina is not yet well understood, it is already clear that their lack of desensitization is a very important feature and one that is highly conserved in evolution, at least from fishes to humans.

In the case of the heteromeric GABA $_A$ receptors, it is known that their rate of desensitization depends on the types of subunits that make up a particular receptor, on the state of phosphorylation of the protein, and on the identity of some amino acid residues located in the second transmembrane segment (TM2) (16, 17). Furthermore, it has been shown that modifications in the TM2 region of some ligand-gated ion channels, including nicotinic and GABA $_A$ receptors, dramatically affect the rate of desensitization and can even change an antagonist substance into an agonist (18–20). In contrast, relatively little is known about the molecular features that confer to GABA $_C$ receptors their comparative lack of desensitization. Here, we examined the possibility that some molecular structural domains are involved in determining the rate of desensitization of ρ 1 receptors. For that purpose, we constructed chimeric DNAs between ρ 1 and the α 1-subunit of the GABA $_A$ receptor. These chimeras were then injected into *Xenopus* oocytes (21) to study the functional properties of the chimeric receptors expressed.

Materials and Methods

Plasmids and DNA Manipulations. For the construction of ρ 1/ α 1 chimeras we cloned a human GABA α 1 receptor subunit from a human brain cDNA library (CLONTECH); the sequence corresponded to that reported by Schofield *et al.* (22). The cloning of GABA ρ 1 has been described previously (7). The high-fidelity DNA polymerase Pfu was used to amplify by PCR the 5' end of GABA ρ 1 from the start codon (forward primer: 5'-ATG AGA TTT GGC ATC TTT CTT-3') to the end of the first intracellular loop (reverse primer: 5'-TCT GCG GTC GAT CCA GAA-3'). GABA α 1 was amplified from the start of the TM2 segment to the end of the coding sequence by using a phosphorylated forward primer (5'-p-GTA CCA GCA AGA ACT GTC TT-3') and a reverse primer (5'-GCA TGC CTA TTG ATG GGG GGT GGG-3'), introducing an *Sph*I site after the end of the coding sequence to facilitate subsequent cloning manipulations. PCR cycling was performed at 94°C (30 sec), 55°C (30 sec), and 72°C (45 sec) for 25 cycles, with a final extension step of 5 min at 72°C. Amplified fragments were visualized in a 1% agarose gel, isolated, and ligated. The products were restricted with *Msc*I and *Sph*I, introduced into the plasmid pAV111, and sequenced. This new chimeric gene will be referred to hereafter as ρ 1/ α 1. Finally, to increase the expression level, the correctly formed chimera was shuttled as a *Bam*HI-*Xho*I fragment into pcDNA3 (Invitrogen).

To swap the TM2 region of ρ 1 for that of the α 1-subunit we used as a PCR template the ρ 1/ α 1 chimera to amplify the upstream segments, from the 5' end to the 3' end of the α 1TM2 segment. This amplicon was ligated to a segment of the 3' end

Abbreviations: GABA, γ -aminobutyrate; TM2, second transmembrane region; wt, wild type; TPMPA, (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid.

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of $\rho 1$ that spanned the first extracellular loop to the 3' end. This chimera, named $\rho 1_{[\alpha 1\text{TM}2]}$, was introduced into pcDNA3 and used for nuclear injection of oocytes. Schematic diagrams of the chimeras are shown in Figs. 1 and 4.

Electrophysiological Recordings in *Xenopus* Oocytes. Isolation of oocytes and recordings were essentially as described previously (21, 23–25). Briefly, ovaries from female *Xenopus laevis* were dissected out, follicles were isolated manually, and, to remove the enveloping follicular cells, the follicles were treated with 0.5 mg/ml collagenase type 1 (Sigma) for 1 h (21) and finally maintained at 16°C in Barth's medium containing gentamycin (0.1 mg/ml). One day later, 5–10 nl of plasmid pcDNA $\rho 1$, pcDNA $\rho 1/\alpha 1$, or pcDNA $\rho 1_{[\alpha 1\text{TM}2]}$ at 0.5 mg/ml was injected into the nucleus of *Xenopus* oocytes. A combination of GABA $_A$ receptor subunits $\alpha 1\beta 2\gamma 2\text{L}$ (2:2:1) was injected at the same concentration (cf. ref. 24). Electrophysiological records were similar to those we have described previously (4, 7, 23–25). Dose-response relations were fitted with the Hill equation (cf. ref. 19). To estimate the rate of desensitization, the GABA currents were fitted with one or two exponential decay functions: τ_s and τ_f are the time constants for the slow- and fast-decay components respectively.

Results

A $\rho 1/\alpha 1$ Chimera That Expresses Fast-Desensitizing Homomeric Receptors. Gene chimeras have been very useful to “dissect” functional protein domains, including those of ligand-gated ion channels. To study the domains responsible for the lack of desensitization of GABA $_C$ receptors, we constructed gene fusions between $\rho 1$ and GABA $_A\alpha 1$. The first ($\rho 1$) part of the $\rho 1/\alpha 1$ chimera is formed by the amino terminus region of GABA $\rho 1$, from the translational start codon to the 3' end of the first intracellular loop at residue 274. The second ($\alpha 1$) portion spans from the start of the TM2 segment of the GABA $_A\alpha 1$ subunit at residue 254 to the end of the coding sequence (Fig. 1A). Upstream and downstream noncoding sequences of $\rho 1/\alpha 1$ are part of a $\rho 1$ cDNA, which originally was cloned from human retina (4). According to the structural model for ligand-gated ion channels, the pore of the channel of the receptor, presumably formed by the TM2 segment, would derive entirely from the GABA $_A\alpha 1$ subunit, whereas the large, extracellular amino-terminal domain, containing the agonist-binding site, would be formed entirely by $\rho 1$.

When oocytes injected with the $\rho 1/\alpha 1$ chimera were exposed to GABA they generated GABA currents whose macroscopic characteristics contrasted sharply with those generated by oocytes injected with the wild-type (wt) $\rho 1$ (cf. Fig. 1B and D). As is well known, $\rho 1$ receptors and its variant $\rho 1\Delta 51$ desensitize very little (1, 4, 7). In contrast, the currents generated by the $\rho 1/\alpha 1$ chimeric receptors were not maintained, and, in the continued presence of high concentrations of GABA, they fell rapidly to a low level. This fast desensitization does not occur for any of the cloned GABA $_C$ subunits ($\rho 1$ – $\rho 3$) expressed in oocytes or other systems (5–9). Furthermore, all our attempts to express functional homomeric $\alpha 1$ receptors, by injecting the wt $\alpha 1$ -subunit alone, gave oocytes that failed to generate clear membrane currents when exposed to as high as 10 mM GABA. Therefore, the desensitizing characteristics of the $\rho 1/\alpha 1$ receptors must be a property of the chimeric protein.

To obtain an estimate of receptor desensitization we measured the time constant of decay of the currents generated by GABA (1 μM) applied to oocytes injected with either wt $\rho 1$, $\rho 1/\alpha 1$, or GABA $_A(\alpha 1\beta 2\gamma 2\text{L})$ DNAs. As already mentioned, the GABA currents of oocytes expressing wt $\rho 1$ receptors desensitize very little during 1–2 min of exposure to GABA. In sharp contrast, the currents generated by the chimeric $\rho 1/\alpha 1$ receptors desensitize strongly and the current decayed along, at

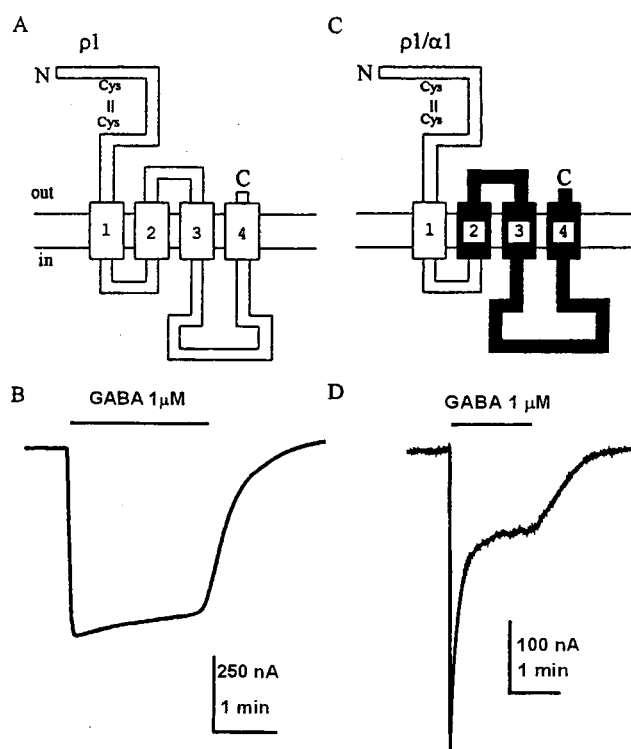


Fig. 1. Diagram of wt $\rho 1$ (A) and $\rho 1/\alpha 1$ chimeric (C) receptors. The $\alpha 1$ portion is shown in black. (B and D) Sample currents elicited by GABA acting on wt $\rho 1$ (B) and $\rho 1/\alpha 1$ (D) receptors. Note the fast desensitization of the chimeric receptor.

least, two exponentials with a $\tau_f = 4.1 \pm 1$ and a $\tau_s = 33.6 \pm 9$ s ($n = 13$), whereas the GABA $_A(\alpha 1\beta 2\gamma 2\text{L})$ receptors showed decay constants of $\tau_f = 7.9 \pm 1$ and $\tau_s = 51.5 \pm 9.3$ s ($n = 7$) for the fast and slow components, respectively. The τ_f of the chimeric receptor was significantly smaller than that of the GABA $_A$ receptor, whereas the τ_s values were not significantly different.

Functional Properties of $\rho 1/\alpha 1$. To see whether other properties of the $\rho 1/\alpha 1$ chimeric receptor correspond to those of a GABA $_C$ or to those of the GABA $_A$ class of receptors, we compared some of their functional characteristics. The GABA dose/current-response relation of the $\rho 1/\alpha 1$ chimeric receptors gave an EC_{50} of 1.06 μM ($n = 15$) (Fig. 2A), i.e., much closer to the EC_{50} (1.02 μM) of $\rho 1$ receptors (cf. also ref. 7) than to the EC_{50} (37 μM) of GABA $_A(\alpha 1\beta 2\gamma 2\text{L})$ receptors (24).

The reversal potential of the currents mediated by $\rho 1/\alpha 1$ receptors was close to -20 mV (Fig. 2B), indicating that the currents are carried mainly by Cl^- ions, as is the case for all $\rho 1$ and GABA $_A$ receptors. In addition, the current-voltage relation of the $\rho 1/\alpha 1$ receptors was essentially linear, that is, similar to that of wt $\rho 1$ receptors and in sharp contrast to that of GABA $_A$ receptors, which show marked rectification at negative potentials (26). Moreover, the overall pharmacological profile of $\rho 1/\alpha 1$ was similar to that of the wt $\rho 1$ receptor. For example, $\rho 1/\alpha 1$ receptors were almost completely resistant to inhibition by bicuculline (up to 1 mM), which contrasts with the potent inhibitory effect of bicuculline on GABA $_A$ receptors ($\text{IC}_{50} = 1.4$ μM) (24), and zinc ions blocked the chimeric receptors with an IC_{50} of 5.5 μM (Fig. 3A), whereas the IC_{50} for wt $\rho 1$ is 22 μM (7). Furthermore, the specific GABA $_C$ antagonist (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid (TPMPA) (27, 28) antagonized the GABA

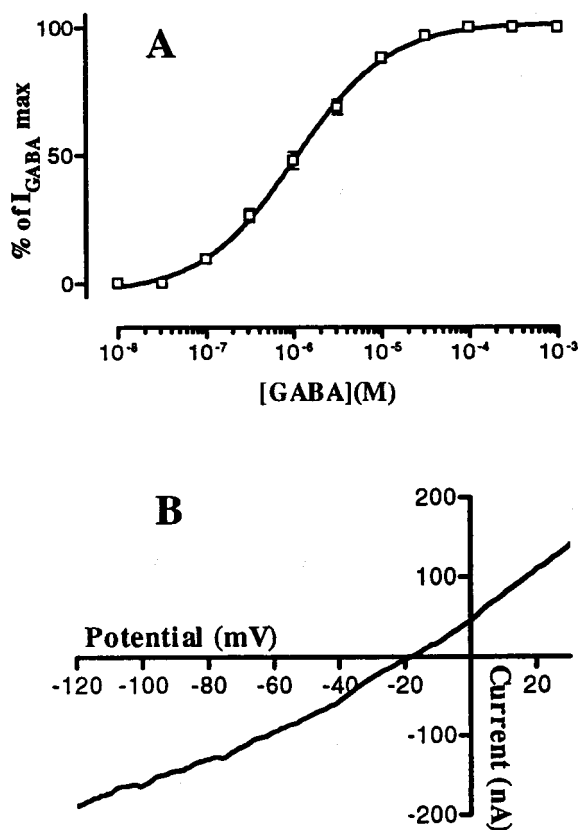


Fig. 2. Properties of $\rho 1/\alpha 1$ receptors. (A) Average dose/response relationship ($n = 15$). (B) Current-voltage relation of one oocyte. Note the weak rectification of chimeric receptors, which is similar to that of wt $\rho 1$ receptors.

currents of $\rho 1$ receptors ($IC_{50} = 1.6 \mu M$) and those of the chimeric $\rho 1/\alpha 1$ receptors with approximately the same potency ($IC_{50} = 1.3 \mu M$) (Fig. 3B), whereas GABA_A receptors were only slightly affected. Actually, at high concentrations of either zinc or TPMPA, the application of GABA elicited an outward current, instead of the usual inward current (Fig. 3). This is probably because at high concentrations, zinc and TPMPA block poorly characterized, inward-resting currents, and this block became evident after the action of GABA on the chimeric receptors was fully blocked.

Transposing the TM2 Region of GABA_A $\alpha 1$ Does Not Confer Fast Desensitization to $\rho 1$. After we found that the desensitizing property of GABA_A receptors could be transferred to $\rho 1$ by swapping their 3' ends, we focused our attention to the TM2 region, which, when mutated, alters the desensitizing kinetics of other ligand-gated ion channels (18–20). However, instead of making single site mutations in the TM2 region of $\rho 1$, we replaced the entire TM2 region of $\rho 1$ by the corresponding segment of the $\alpha 1$ -subunit, thus making a $\rho 1_{[\alpha 1 TM2]}$ chimeric receptor that includes the entire $\rho 1$ receptor except for its TM2 region (Fig. 4A). This construct expressed functional receptors that elicited GABA currents that did not desensitize (Fig. 4B). That is, in this respect, the $\rho 1_{[\alpha 1 TM2]}$ chimeric receptor behaved like the wt $\rho 1$ receptor; but the GABA currents of oocytes expressing the $\rho 1_{[\alpha 1 TM2]}$ chimeric receptor were more than 100 times smaller than those of oocytes expressing wt $\rho 1$ receptors, and many oocytes failed to respond to GABA. Despite all this, the $\rho 1_{[\alpha 1 TM2]}$ GABA currents recorded from 12 oocytes (mean,

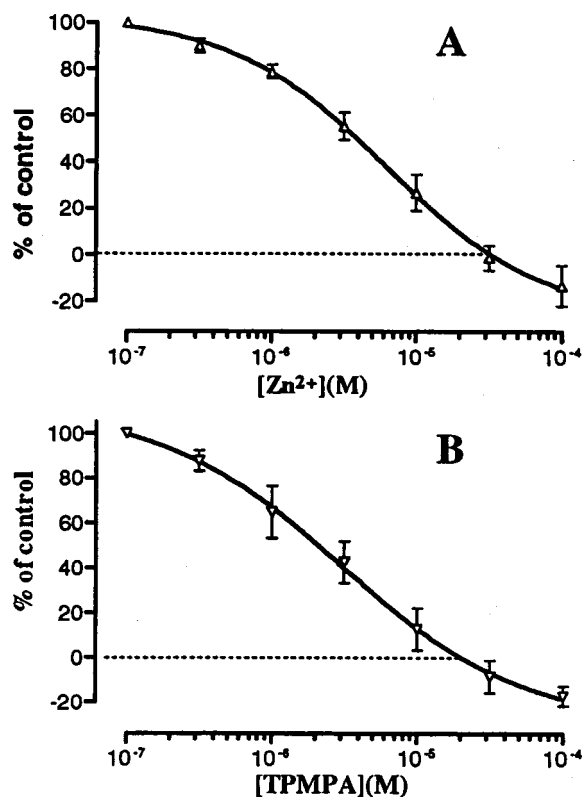


Fig. 3. Inhibition of GABA currents generated by $1 \mu M$ acting on GABA $\rho 1/\alpha 1$ receptors. (A) Block by zinc. Data show means \pm SE. (B) Antagonism by TPMPA ($n = 4$).

26 nA) were sufficiently large to allow a gross characterization of these chimeric receptors. Thus, the EC_{50} for GABA was $0.3 \mu M$ (Fig. 4C), and the chimeric receptors were insensitive to bicuculline (up to 1 mM) and were antagonized by TPMPA (IC_{50} ca. $1 \mu M$) (Fig. 4D).

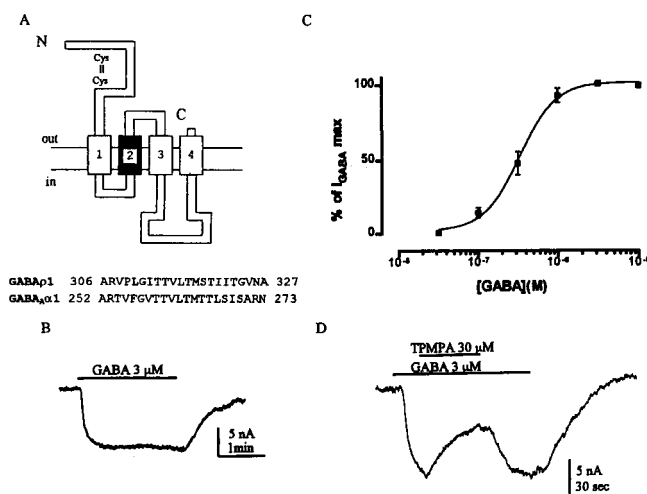


Fig. 4. Properties of a chimeric $\rho 1$ receptor with its TM2 segment replaced by that of the GABA_A $\alpha 1$ -subunit. (A) Diagram of the construct. The $\alpha 1$ portion is in black. (B) Sample current response showing lack of desensitization. (C) Average dose/response relationship ($n = 4$). (D) Antagonism of the $\rho 1_{[\alpha 1 TM2]}$ receptors by TPMPA.

Discussion

A very important characteristic of neurotransmitter receptors is that, during the continuous application of an agonist, their response is not maintained but decays rapidly to a low level. This phenomenon is generally known as desensitization and it almost certainly involves many different processes. Most neurotransmitter receptors desensitize, especially when exposed to high agonist concentrations, but there are two receptors that show little desensitization: one of them is the kainate receptor and the other is the recently discovered GABA_C receptor (1–5). The latter is particularly interesting because a single type of subunit (ρ) forms homooligomeric receptors, whereas other members of the family of GABA receptors are heteromeric and well known to show marked desensitization. Nonetheless, the mechanisms responsible for this desensitization remain essentially unknown. The present experiments represent our initial attempts to elucidate the molecular mechanisms involved in the nearly complete lack of desensitization of GABA_C receptors.

Analysis of gene chimeras in combination with the oocyte expression system provides a powerful model to elucidate functional motifs in receptor ion channels. Therefore, to begin to define the protein domains involved in the desensitization mechanisms, we constructed $\rho 1$ -GABA_A $\alpha 1$ chimeric genes, taking advantage of the presumed homooligomerization signal (29) located in the extracellular N terminus segment of $\rho 1$. This signal, a putatively glycosylated asparagine residue, allowed the formation of functional homomeric chimeric receptors. This contrasts sharply with the difficulty of forming homomeric GABA_A receptors. The most important trait of the $\rho 1/\alpha 1$ chimera GABA currents is the appearance of a fast-desensitization component. Thus, it is clear that a large part of the $\alpha 1$ receptor (spanning from the TM2 segment up to the carboxyl terminus end of the protein) is sufficient to transfer this typical GABA_A characteristic to a nondesensitizing receptor, such as $\rho 1$. It should be noted that the ion channel of the chimeric receptor is formed, putatively, by the TM2 segment of $\alpha 1$, and it is known that this region is involved in the process of desensitization of various receptors, including GABA_A and nicotinic receptors (18–20). For example, the rate of desensitization of heteromeric GABA_A receptors is increased when they are formed by GABA_A $\alpha 6$ -subunits with a single point TM2 mutation and the $\beta 2$ - and $\gamma 2s$ -subunits (18). Therefore, it is not surprising that the $\rho 1/\alpha 1$ chimeric receptors showed desensitization. What is surprising is that replacing the TM2 segment of $\rho 1$ by that of an α -subunit did not confer desensitization to the receptors expressed. Therefore, the TM2 segment is not the only, and perhaps not even the most important, receptor domain involved in the process of desensitization.

After all this work had been completed it was reported that a single amino acid change in the TM2 segment of the perch $\rho 1$ receptor accounts for its desensitization kinetics (30). In that case, a proline residue was substituted by serine and desensitization increased. In the case of our $\rho 1_{[\alpha 1\text{TM}2]}$, the

presence of the GABA_A $\alpha 1$ TM2 region did not increase the rate of desensitization. A comparison of the amino acid sequences of these receptors shows that the proline located in the perch $\rho 1$ TM2 segment is conserved in the same position of the human $\rho 1$, whereas in the $\rho 1_{[\alpha 1\text{TM}2]}$, that proline is substituted by a valine. Furthermore, the transfer of the TM2 segment changed six amino acids: four of them were changed to amino acids with similar, nonpolar side chains (P309V, I312V, S319T, I321L), and the other two resulted in changes of nonpolar for uncharged polar residues (V308T and I322S). Apparently, none of these changes altered the desensitization of the chimeric receptor. However, there was a clear decrease in the magnitude of the GABA currents elicited. So far, we do not know whether this was due to an inherent property of the receptors expressed or to a lower number of receptors in the membrane, etc. Nevertheless, altogether our results suggest that, at least for $\rho 1$, the kinetics of desensitization are not determined by a single site, or domain, but are determined instead by an interaction of several sites distributed along the receptor.

TPMPA is a hybrid of isoguvacine and 3-APMPA that was designed as a strong competitive antagonist of GABA_C receptors, with a more than 100-fold selectivity for GABA_C receptors as compared with GABA_A or GABA_B (27, 28). Therefore, it is interesting that the $\rho 1/\alpha 1$ chimeric receptors retained the TPMPA sensitivity, suggesting that the extracellular amino terminus domain of $\rho 1$ is involved principally in the recognition site for this compound. Moreover, bicuculline, which blocks GABA_A but not GABA_C receptors, also was ineffective on the $\rho 1/\alpha 1$ chimeric receptors. This suggests again that the bicuculline resistance is conferred mainly by the extracellular amino terminus domain of $\rho 1$.

There is increasing evidence indicating that zinc plays a major role in the modulation of ligand-gated ion channels including GABA_A, GABA_C, and nicotinic receptors. We have characterized previously the effects of zinc on $\rho 1$ as well as on its alternative spliced form, $\rho 1\Delta 51$ (7, 25). Here, we show that zinc was even a more potent blocker of the chimeric $\rho 1/\alpha 1$ receptors than of the wt $\rho 1$ receptors. This is consistent with the characterization of a zinc-binding site in the amino terminus of $\rho 1$ by Wang *et al.* (31), who showed that histidine 156 was essential for zinc modulation of $\rho 1$ receptors. Our results thus support their findings because $\rho 1/\alpha 1$ receptors retained the ability of being modulated by zinc, presumably because of the conservation of histidine 156. However, the increased sensitivity of the chimeric $\rho 1/\alpha 1$ vs. the wt $\rho 1$ receptor still remains to be explained.

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